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(54) Title: A VACCINE AGAINST TOXOPLASMA GONDII

(57) Abstract

Vaccines which are capable of providing protection against infection with *Toxoplasma gondii* are provided. Methods for immunizing healthy animals against *Toxoplasma gondii* infection are also provided.

Reference B

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A VACCINE AGAINST *TOXOPLASMA GONDII*

Background of the Invention

Toxoplasma gondii, an obligate intracellular protozoan parasite, is perhaps the most prevalent source of parasitic infection of humans in the world, although only a limited number of individuals actually become symptomatic with the infection. Infection with *T. gondii* is primarily a concern to the newborn and individuals who are immunocompromised. In the United States alone, over 3000 cases of congenitally acquired Toxoplasmosis occur annually. This infection gives rise to a wide range of neurological abnormalities including hydrocephalus, mental retardation and blindness. In addition, children afflicted with this disease have decreased performance in school and frequently suffer from seizures. In adults the major group of individuals suffering from Toxoplasmosis are those with AIDS. It is currently estimated that in the United States alone between 15-22% of the individuals having AIDS will develop Toxoplasmic Encephalitis (TE) during the course of their illness. The prevalence in Europe is even higher.

T. gondii also infects other mammals and birds. This parasite causes blindness in cats, the definitive host for the parasite. It also is a major cause of abortion in cattle and sheep making it a major economic concern to the agriculture industry. Domestic animals which carry this infection can also pass it on to humans in undercooked meat.

Infection of the immunocompromised or newborn results in the necessity for long term chemotherapy that is frequently not

well-tolerated because of bone marrow toxicity and allergic reactions. Accordingly, there exists a need for the development of effective prophylaxis against this parasite.

An attenuated live vaccine is currently available for use in livestock. However, animals vaccinated with this vaccine remain infected with *Toxoplasma* and are capable of transmitting the disease even though the organism is attenuated. A cat vaccine is also currently under development which utilizes a live parasitic strain which is unable to produce infectious oocysts. Cats vaccinated with this vaccine develop systemic immunity and cannot be infected with a wild-type strain. However, to be effective this vaccine needs to be administered to both domestic and feral cats. Further, the parasite itself still remains highly infectious so that any breakthrough could result in an outbreak of the disease.

It has now been found that a non-human pathogen, *Neospora*, has the ability to protect animals against *Toxoplasma* infection.

Summary of the Invention

An object of the present invention is to provide vaccines comprising a non-human pathogen, *Neospora*, which are capable of providing protection against infection with *Toxoplasma gondii*.

Another object of the present invention is to provide a method of immunizing healthy animals against *Toxoplasma gondii* infection comprising administering to a healthy animal an effective amount of a vaccine comprising a non-human pathogen, *Neospora*.

Detailed Description of the Invention

The present invention provides a vaccine against *Toxoplasma* infection in animals. This vaccine comprises the non-human pathogen *Neospora* which is used as a live, attenuated vaccine against *Toxoplasma* infection in domestic animals. In addition to protecting these animals from *Toxoplasma*, this vaccine also serves as a transmission blocking vaccine for

humans, since preventing disease in livestock indirectly reduces infection in humans.

The *Neospora* parasite was originally isolated from dogs. Dubey et al. *J. Parasit.* **1990** 76:732-4; Hay et al. *J. Am. Vet. Med. Assoc.* **1990** 197:87-9; Dubey et al. *Vet. Rec.* **1990** 126:193-4; Dubey et al. *J. Parasit.* **1990** 76:127-30. This parasite was reported to cause a spastic paralysis of the hind limbs of newborn puppies. This organism is a *Coccidia* and a member of the genus, *Apicomplexa*. Phylogenetically, it appears quite similar to *T. gondii*, although studies indicate that *Neospora* does not express the major surface antigen of *T. gondii*, SAG1, nor does it appear to contain the gene for expression of the SAG1 molecule. The SAG1 surface antigen has been identified on all *T. gondii* isolates from various parts of the world.

This *Neospora* organism does not appear to be a human pathogen. Further, there is no evidence of infection in pigs and only one reported case of abortion in sheep. A number of cattle have been found to be infected with *Neospora*. However, pigs and sheep are the major source of *Toxoplasma* infection in man.

Despite the absence of evidence for natural human infection, it has been found that the this *Neospora* parasite readily infects human cells including cells of the myeloid and epithelial origin. Further macrophage derived monocytes that are infected with this parasite respond by producing soluble immune factors that can alter the host response.

Studies in mice demonstrate that infection with *Neospora* is able to protect both inbred and outbred mice 100% against a lethal challenge with *Toxoplasma*. Complete protection against *Toxoplasma* was observed as soon as six days post *Neospora* infection and is continued to be seen out to 3 months following *Neospora* infection.

Adoptive transfer experiments have also been performed. Naive mice were immunized with immune splenocytes obtained from 14 day post *Neospora* infected mice and then exposed to a lethal challenge of *T. gondii*. Almost 90% of the mice survived challenge as compared to no survivors in the non-transferred

groups thus indicating that T cell immunity is an important component of this cross reactive protective immune response.

Immunofluorescence assays have been used to determine that sera raised against *Neospora* weakly cross reacts with *T. gondii*. In contrast, *Toxoplasma* antisera is strongly reactive against *Neospora* indicating that there are shared immune epitopes between the two organisms that may differ in the degree of exposure to antibody binding. Western blot analysis confirms several cross reactive bands between the two organisms.

Further studies have demonstrated that *N. caninum* stimulates a strong TH1 like response. Th1 cytokines, such as IFN_γ, IL-2, IL-12 and IL-7, are important in host immunity to Toxoplasma. Exogenous administration of these cytokines can protect mice against lethal *T. gondii* challenge. Neutralization of endogenous IL-12 in *Neospora* infected mice was found to lead to the development of symptomatic illness and finally death. Message for IFN-γ was shown to increase in all mice infected with *Neospora* as compared to controls. Thus, it is believed that the development of the TH1 like response may be an essential component of the cross reactive host response observed.

These studies demonstrate that *Neospora* can be incorporated into a vaccine and administered to a healthy animal in an effective amount to protect against infection by *Toxoplasma*. "Effective amount" refers to that amount of vaccine which invokes in an animal an immune response sufficient to provide protection against a lethal challenge of *Toxoplasma*. By animal, it is meant any mammal or bird which can be infected by *Toxoplasma*. In a preferred embodiment, the vaccine is directed at domestic livestock to prevent primary infection in pigs and sheep. It is also believed that the vaccine will prevent congenital transmission as well. Pigs and sheep are both sources of *Toxoplasma* infection in man. Dubey, JP, *J. Vet. Diagn. Invest.* **1990** 2:230-3; Dubey JP, *J. Parasit.* **1990** 76:127-30. Alternatively, the vaccine can be administered to cats. By preventing Toxoplasmosis in livestock and

domestic cats, transmission to humans, especially women of child bearing age can be significantly reduced. Accordingly, the vaccine of the present invention also serves as a transmission blocking vaccine.

Neospora can be suspended in any pharmaceutically acceptable carrier suitable for injection. Examples of pharmaceutically acceptable carriers include, but are not limited to, normal isotonic saline, standard 5% dextrose in water or water. The vaccine can be administered subcutaneously or intraperitoneally.

Alternatively, a recombinant vaccine can be prepared by culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a *Neospora* antigen, under conditions promoting expression of this antigen followed by subsequent recovery of this antigen and incorporation into a vaccine preparation. In this recombinant vaccine, the *Neospora* antigen is an antigen capable of producing a cross reaction with *T. gondii*. Recombinant preparation of the *Neospora* antigen is performed using techniques well-known to those skilled in the art.

This invention is further illustrated by the following nonlimiting examples.

EXAMPLES

Example 1: Protection Studies in Mice

Protection studies were evaluated in both inbred (A/J, Balb/c) and outbred (CD-1) 5 to 6 week old female mice. The first group (n=8 mice) were infected intraperitoneally with 1 x 10^6 tachyzoites of *N. caninum* or saline. After one month, mice were challenged with *T. gondii* tachyzoites. For the A/J mice the challenge dose was 2 x 10^4 parasites whereas Balb/c mice received 1 x 10^4 *T. gondii* tachyzoites. All control mice died by day 10 post infection. All of the mice infected with *Neospora* survived the lethal *T. gondii* challenge. These experiments were repeated three times and similar observations were obtained.

Example 2: Determination of Duration of Host Protection

Mice were infected with *N. caninum* as described in Example 1 and challenged with *T. gondii* at progressively increasing intervals post-*Neospora* infection out to three months post infection. The protective response remained unchanged to at least three months post infection.

Example 3: Dose Determination

A study was performed to establish the required dose of *Neospora* which stimulates immunity against *Toxoplasma*. Outbred strain (CD-1) mice were infected with *Neospora* (1×10^6, 5×10^5 and 5×10^4) as described in Example 1. Two weeks post infection, the mice were challenged with a lethal inoculum of P strain tachyzoites of *Toxoplasma* (2×10^4). An inoculum of 1×10^6 *Neospora* was demonstrated to provide 100% protection against *Toxoplasma* challenge. Infection with 5×10^5 *Neospora* protected 75% (6/8) and mice infected with 5×10^4 *Neospora* failed to exhibit significant protection against subsequent *T. gondii* infection. Alternatively, all mice inoculated with 1×10^6 *Neospora* and challenged with 2×10^4 *Toxoplasma* survived. Protection was reduced to 50% when the challenge dose was increased to 1×10^5 *T. gondii* tachyzoites.

Example 4: Adoptive Transfer Experiments

Inbred A/J mice were immunized with *Neospora* as described in Example 1. At day 14 post infection, the splenocytes were isolated and transferred (1 x 107) via intravenous inoculation into naive recipient mice. The recipients were challenged with a lethal dose of *Toxoplasma*. Almost 90% (5/6 mice) survived challenge compared to no survivors in the non-transferred group.

Example 5: Cytokine Studies

A/J mice were infected with 1×10^6 *Neospora* tachyzoites. The infected mice were then treated with 0.5 mg of goat anti-mouse IL-12 twice weekly to neutralize endogenous IL-12. Control animals were treated with equal amounts of goat IgG.

Mice were treated with anti IL-12 one day before the challenge with a lethal dose of *T. gondii* and treatment was continued twice a week. By day seven post infection, all mice depleted of IL-12 were dead. In contrast, mice treated with the isotype control antibody showed no signs of sickness and survived the entire observation period. The histopathology of various tissue of the IL-12 depleted mice showed extensive intracellular replication of *N. caninum*, with necrosis and little inflammation.

Example 6: Cytokine Message Levels

A/J mice were infected with either *Neospora* or saline and challenged with 2 x 10^4 P strain tachyzoites of *T. gondii*. One week after challenge the splenocytes were isolated and RNA extracted using TRIzol. Reverse transcription was performed using murine MMLV RT (GIBCO, BRL, Bethesda, MD) and random hexamer primers (Promega, Madison, WI). For PCR, a multiple cytokine-containing competitive construct PQRS was utilized. Aliquots of cDNA were assayed for hypoxanthine-guanine phosphoribosyltransferase (HPRT), IL-2, IFN-γ, IL-10 and IL-12 by examining the ratio of competitor-to-wild-type band intensity following amplification of each primer set. After cycling, separation of the PCR products was accomplished by electrophoresis on 3% agarose gel. This separation allowed for the discrimination of the larger competitor-construct from the unknown, wild-type cDNAs that migrate at a faster rate in the gel.

What is claimed is:

1. A vaccine for protection of an animal against infection with *Toxoplasma gondii* comprising a *Neospora* antigen.

2. The vaccine of claim 1 wherein the *Neospora* antigen comprises live, attenuated *Neospora*.

3. The vaccine of claim 2 further comprising a pharmaceutically acceptable carrier.

4. The vaccine of claim 1 wherein the *Neospora* antigen is recovered from host cells containing a nucleic acid sequence encoding a *Neospora* antigen.

5. The vaccine of claim 4 further comprising a pharmaceutically acceptable carrier.

6. A method of protecting an animal against *Toxoplasma gondii* infection comprising administering to an animal an effective amount of a vaccine comprising a *Neospora* antigen.

7. The method of claim 6 wherein the *Neospora* antigen of the administered vaccine comprises live, attenuated *Neospora*.

8. The method of claim 7 wherein the vaccine further comprises a pharmaceutically acceptable carrier.

9. The method of claim 6 wherein the *Neospora* antigen of the administered vaccine is recovered from host cells containing a nucleic acid sequence encoding a *Neospora* antigen.

10. The method of claim 9 wherein the vaccine further comprises a pharmaceutically acceptable carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18100

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 39/02
US CL :424/234.1; 530/300
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/234.1; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, WPIDS
search terms: Neospora, Toxoplasma gondii, toxoplasmosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/25541 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 September 1995 (28-09-95), see entire document, especially pages 3 and 21-23.	1-10
X	LINDSAY et al. Infection of Mice with Neospora caninum (Protozoa: Apicomplexa) Does Not Protect against Challenge with Toxoplasma gondii. Infection and Immunity. August 1990, Vol. 58, No. 8, pages 2699-2700, see entire document.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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